

## Specification

Method for controlling mycotoxin contamination of cereals

## 5 Technical Field

The present invention relates to a treatment method which intends to reduce contamination amount of mycotoxin produced by plant pathogenic fungi of cereals (hereinafter represented as "DON") in the cereals by using a composition  
10 for agri-horticulture containing one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester as an active  
15 ingredient(s).

## Background Art

Fusarium head blight in cereals frequently occurs in cases of continuous cloudy weather and light rain, and  
20 furthermore high temperature from the heading date to the milk-ripe stage (Yukio Ozeki, Hiroshi Sasaki, Yoichi Amano, Hokkaido no Hatasaku Gijutu (Farming Technology in Hokkaido) -Cereals version-, published by Society for Agricultural Technique Propagation, page 209, 1978), and it  
25 is a plant disease which causes great damage to cereals in terms of yields, quality and so forth, and can not be avoided in this country due to the high amount of rainfall during the ripening period. As major pathogenic fungi thereof, *Fusarium graminearum*, *Fusarium culmorum*, *F.*  
30 *avenaceum* and *Microdochium nivale* have been specified. Although there is difference in occupancy depending on climate conditions and areas, complex infection is often observed in farm fields where the disease has occurred (Kunihei Kishi, Nippon Shokubutsu Byogai Daijiten  
35 (Comprehensive Dictionary of Plant Disease in Japan), published by Zenkoku Noson Kyoiku Kyokai (Association for

National Farming Village Education), page 74, 1998).

The plant pathogenic fungi which cause this disease produce more than one toxic metabolites referred to as mycotoxin, which contaminates crops during cultivating and poses a risk of ingestion by human and domestic animals through migration to harvestries and processed foods. Researches on mycotoxin in areas such as Europe, North America and East Asia where cultivation of the cereals is active have long history, DON has been specified as a toxic agent whose effects on human and animals are most concerned in terms of both toxicity and the contamination amount, and noticed worldwide. Ingestion of foods contaminated with DON causes acute poisoning where digestive organ symptoms including emesis and diarrhea are major symptoms. In Europe and North America, self-regulating values of DON contamination amount in grains have been established, and the system for intensified surveillance has been organized. While internationally rising momentum in DON surveillance, in 2002 in this country, the Ministry of Health, Labor and Welfare also established an interim standard value of 1.1 ppm for the DON contamination amount in the wheat, and announced that safety of the wheat distributed in the market was assured (Shokuhatu No. 0521001). In relation to the announcement, as an instruction notice for feed safety, the Ministry of Agriculture, Forestry and Fisheries established the interim acceptable value 4.0 ppm for deoxynivalenol in feeds supplied for cattle aged 3 months or more and 1.0 ppm that for domestic animals other than the above (press release). Before DON has been noticed as a fungus producing toxin, people have been protected from healthy risk due to fungous toxin by a law regulation that the contamination ratio of the mixed grains affected with Fusarium head blight shall be made less than 1% by visual check at the shipping stage of crude wheat. Thus, in the production fields of the cereals, the damages with Fusarium head blight have been reduced and inhibited by giving

fungicidal agents effective for pathogenic fungi of Fusarium head blight.

Chemicals effective for the pathogenic fungi of Fusarium head blight of the cereals commonly used at present are classified into several groups by their action mechanisms and chemical structures of active ingredients (Agricultural Chemical Handbook, 2001 version, published by Nippon Shokubutsu Boeki Kyokai). The SBI agents characterized by inhibition of biosynthesis of sterol universally present as a component of biological membrane of fungi include (RS)-1-p-chlorophenyl-4, 4-dimethyl-3-(1H-1,2,4-triazole-1-ylmethyl) pentane-3-ol (generic name: tebuconazol), (1RS, 5RS)-5-(4-chlorobenzyl)-2, 2-dimethyl-1-(1-H-1,2,4-triazole-1-ylmethyl) cyclopentanol (generic name: metconazol), and 1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolane-2-ylmethyl]-1H-1,2,4-triazole (generic name: propiconazol), and they are characterized by having a triazole skeleton in the chemical structures. They have been widely used since they are effective at low doses, rapidly permeate in plant bodies, are resistant to rain and low toxic for flower-visiting insects, and they have a high protection effect on the pathogenic fungi of Fusarium head blight of the cereals. Methyl=(E)-2-(2-[6-(2-cyanophenoxy)pyridine-4-yloxy]phenyl)-3-methoxyacrylate (generic name: azoxystrobin) and methyl=(E)-2-methoxyimino [ $\alpha$ -(o-tolyloxy)-o-tolyl] acetate (generic name: kresoxim-methyl) which were developed as strobilurine derivatives which are anti-mycotic antibiotics and structurally characterised by methoxyacrylate ester are classified into methoxyacrylate type fungicidal agents. The former exerts the protection effect by the inhibition of respiratory activity of the bacteria, and the latter does it by the inhibition of cytochrome electron transfer system in mitochondria. The other synthetic fungicidal agents include 1,1'-iminodi(octamethylene)diguanidium=triacetate (generic name: iminooctadine acetate). It is considered as the action

mechanism that it causes destruction of a membrane lipid bilayer structure of bacteria due to a surfactant-like feature derived from its salt structure. Furthermore, the relevance between the pathogenic fungi and drug efficacy has been researched, and it has been demonstrated that the triazole agents are effective for the 3 species of *F. graminearum*, *F. culmorum* and *F. avenaceum*, and that the methoxyacrylate agents are effective for *M. nivale*. As mentioned above, occurrence of the disease often accompanies mixture of the multiple pathogenic fungi, and thus, the occurrence of the disease has been protected and inhibited by performing rotation spray with taking advantage of characters of respective agents.

Along with attention for DON, as analysis of its contamination concentration has progressed, it has been being demonstrated that there is no direct relevance between an illness degree of the Fusarium head blight and the contamination amount of DON (Bai, G. H., Plattner R and Desjardins A., Relationship between visual scab rating and deoxynivalenol in wheat cultivars, The 1988 National Fusarium Head Blight Forum, Chapter 2, pages 21-25). It has been also demonstrated in recent years that *F. graminearum* and *F. culmorum* have DON production capacity but *F. avenaceum* and *M. nivale* do not produce DON. It has been reported that when *M. nivale* is controlled using the methoxyacrylate agent, *F. graminearum* and *F. culmorum* which are antagonized are increased resulting in facilitating the increase of DON contamination amount. Thus, the disease protection only by giving the fungicidal agents can not sufficiently prevent the DON contamination. Furthermore, in the production fields of the cereals, even when the Fusarium head blight of the cereals is controlled by combined application of various fungicidal agents, DON is frequently detected at more than 1.1 ppm. This troubles the producers. That is, the DON contamination amount can not be sufficiently reduced only by controlling the

pathogenic fungi having a mycotoxin production capacity, which is a conventional technology. Also with respect to fosetyl described in the present statement, the fungicidal effect on the plant pathogenic fungi has been known  
5 publicly (US Patent No. 4,139,616, 1979; and JP 62-87504 A), but there is no mention at all for effects on plants contaminated with mycotoxin. Under such a circumstance, it is an actual state that establishment of a method of substantially reducing the DON contamination amount has  
10 been strongly desired in the production fields of the cereals.

The present inventors studied on compositions for agriculture which reduces contamination of wheat with mycotoxin, particularly a DON contamination amount to 1.1  
15 ppm or less or a low contamination amount as possible. As a result, a composition for agri-horticulture developed using one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal  
20 salts and polyvalent metal salts of phosphorous acid and phosphite ester as an active ingredient(s) has a low controlling effect on pathogenic fungi of Fusarium head blight of the cereals but has an excellent contamination inhibitory effect on a mycotoxin contamination,  
25 particularly the DON contamination, and have completed the invention.

Also, the inventors have shown a reduction of the DON contamination amount equal to or less than the standard value in mixed use with a fungicidal agent where DON  
30 contamination at high concentration of more than the standard value 1.1 ppm has been observed in the mixed use with fungicidal agent for agri-horticulture, and a further inhibitory effect on the DON contamination amount in the mixed use with a fungicidal agent which originally shows a  
35 low contamination level, have found an effect where the DON contamination amount is further reduced compared to a

single treatment of a fungicidal agent for agri-horticulture, and have completed the invention.

#### Disclosure of the Invention

5       The present invention is a method of reducing the contamination amount of mycotoxin (particularly deoxynivalenol) in cereals characterized in that one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts,  
10   alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester are given to the cereals.

      Also, the invention relates to a method of reducing a contamination amount of mycotoxin (particularly  
15   deoxynivalenol) in cereals characterized in that a combination of the above compound A is given to the cereals in combination with (suitably as a composition containing both as active ingredients) one or more fungicidal active components for agri-horticulture (hereinafter referred to  
20   as the compound B).

#### BRIEF DESCRIPTION OF THE DRAWINGS

      Fig. 1 represents test results of inhibitory effects on DON contamination by phosphite derivatives and alkyl  
25   phosphite derivatives (Example 1).

      Fig. 2 represents test results of mixed use effects of potassium phosphite with various fungicides (Example 3).

#### Best Mode for Carrying Out the invention

30       Hereinafter, the invention is described in detail  
      The ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester, derived from phosphorous acid are not particularly  
35   limited so long as they reduce the contamination with mycotoxin, particularly DON, and for example, include

alkali metal salts and polyvalent metal salts of phosphorous acid and phosphite ester, and are suitably potassium phosphite and an aluminium salt (generic name: fosetyl) of tris(ethylphosphonate).

5       Methods of assessing the inhibitory effect on mycotoxin contamination, particularly DON contamination in wheat of the invention can includes a method of determining or measuring by comparing a contamination concentration of DON, incidence rate of Fusarium head blight for panicles  
10   and incidence rate for spikelets in wheat, between a group where a single treatment with phosphorous acid and phosphite ester derivative was given, and a group where a treatment with mixed use of other fungicidal composition was given, and a control group without the treatment with  
15   drug, of the wheat cultivated in the field.

It has been confirmed by such a method that the ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite  
20   ester, derived from phosphorous acid have an excellent inhibitory effect on the mycotoxin contamination, particularly DON contamination, independently on controlling of the pathogenic fungi of the cereals.

The compound B in the invention could be a usual  
25   fungicidal active compound for agri-horticulture, and is suitably the fungicidal active compound effective for wheat Fusarium head blight, such as a sterol biosynthesis inhibitor (SBI) having a triazole skeleton, azoxystrobin, kresoxim-methyl and iminoctadine.

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#### EXAMPLES

By citing below test examples using potassium phosphite and an aluminium salt (generic name: fosetyl) of tris(ethylphosphonate) as the compounds A which are the  
35   active ingredients of the invention, the invention is more specifically described, but the invention is not limited

thereto.

Example 1: Single use ( $\pm$  conventional control) effect

Wheat (cultivar: Haruyutaka) was seeded on April 19, 2002, and cultivated according to a conventional  
5 cultivating standard (Hokkaido, Agriculture Department, 1995) to establish test sections with 6.75 m<sup>2</sup> per section (3 repeats). As the compounds A, potassium phosphite and an aluminium salt (generic name: fosetyl) of tris(ethylphosphonate) were given, water solution of 0.038  
10 to 0.120 % in P<sub>2</sub>O<sub>5</sub> was prepared, and 100 L per 10 a was sprayed on leaves at next growth stage. That is, the first spray (June 28, flowering date), the second spray (July 8, 10 days after the flowering date, and the third spray (July 18, 20 days after the flowering date) were performed. A  
15 conventional control for the purpose of controlling plant pathogens and plant insects was performed together. That is, the first [June 20, azoxystrobin (2000 times dilution) + fenitrothion (1000 times dilution)], the second [tebuconazol (June 28, 2000 times dilution) + sumithione  
20 (1000 times dilution)], and the third [July 8, propiconazol (2000 times dilution) + fenitrothion (1000 times dilution)], the forth [July 17, tebuconazol (2000 times dilution) + fenitrothion (1000 times dilution)] were performed. As controls, the test section where only the conventional  
25 control was given and the test section without any control were established. Harvest was performed by harvesting the wheat in 4 m<sup>2</sup> in the test sections on August 9 (52 days after the heading date). After the harvest, the wheat was applied on a rice grader with 2.2 mm opening of screen to  
30 make selected grains, which were then pulverized by a rapid pulverizer to make whole grain powder. The whole grain powder was used as a sample for analysis. The DON contamination concentration was analyzed by ELISA method using commercially available Rida Screen Fast Don supplied  
35 from r-Biopharm.

Preparation of analysis sample solutions and analysis



procedure are briefly described.

- To 5 g of the whole grain powder, 100 ml of water was added, and vigorously agitated for 10 min to make a DON extract solution.

5        - The DON extract solution was centrifuged at high speed, and the supernatant was used for the ELISA analysis.

- According to the method described on ELISA kit, various reagents were added, and subsequently an absorbance of each test solution was measured.

10       - The DON concentration of each test solution was read out from a standard curve made using DON standard solutions.

- The incidence rate of Fusarium head blight for panicles was calculated by counting the number of panicles in 1 m<sup>2</sup> of each section and the number of diseased panicles included therein.

The results of the present test are shown in Table 1.

Table 1. Inhibitory effect of phosphite derivative and alkyl phosphite derivative on DON contamination (2002, Haruyutaka)

	Treatment			DON concentration (ppm)	Incidence rate of Fusarium head blight for panicles(%)
	Active ingredient A	Concentration	Control system		
1	Potassium phosphite	0.038%	Conventional control	2.41	0.3
2		0.070%	Conventional control	0.59	0.0
3		0.112%	Conventional control	0.39	0.9
4			No control	0.69	1.2
5	Fosetyl	0.120%	Conventional control	0.84	0.0
6	Potassium phosphate	0.112%	Conventional control	4.66	0.0
7			Conventional control	6.16	0.9
8			No control	8.69	4.9

Note) The concentration of the active ingredient A is the concentration converted into  $P_2O_5$ .

Note) The incidence rate of Fusarium head blight for panicles was calculated by counting the number of panicles in 1 m<sup>2</sup> of each section and the number of diseased panicles included therein.

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DON was detected at highly contaminated levels which were much higher than the interim standard value, 1.1 ppm that the Ministry of Health, Labor and Welfare had presented from the section with no control and the section with conventional control alone using the fungicidal composition for controlling of Fusarium head blight of the cereals. On the other hand, inhibition of DON contamination depending on treated concentrations was observed in the sections treated with potassium phosphite and the aluminium salt (generic name: fosetyl) of tris(ethylphosphonate), and their contamination levels were

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lower than 1.1 ppm. It is worthy of special mention that even in the section without any control where the conventional control is not performed, the DON contamination concentration is sufficiently reduced by giving potassium phosphite. Furthermore, even when potassium phosphate which is a salt of normal phosphoric acid is given, the DON inhibitory effect is low, and thus it is obvious that the phosphite and alkyl phosphite derivatives play an important role. Even when the incidence rate of Fusarium head blight for panicles was at almost the same degree, DON at high concentration was detected in the section without treatment with potassium phosphite. This way, it is evident that the concentration of DON contamination is reduced by the treatment with phosphorous acid and alkyl phosphorous acid and the derivatives thereof.

Example 2: Toxin-inhibitory effect by seeds diseased with Fusarium head blight in Example 1

Healthy grains and grains diseased with Fusarium head blight were screened from the crude wheat obtained in Example 1, and the DON contamination concentration of the whole grain powder thereof was analyzed by the ELISA method in the same way as shown in Example 1.

The results of the present test are shown in Table 2.

Table 2. DON contamination concentration of grains diseased with Fusarium head blight

	Treatment content			DON concentration (ppm)	
	Active ingredient A	Concentration	Control system	healthy grain	diseased grain
1	Potassium phosphite	0.038%	Conventional control	0.05	76.6
2		0.070%	Conventional control	0.04	44.7
3		0.112%	Conventional control	0.04	25.4
4			No control	0.04	75.6
5	Fosetyl	0.120%	Conventional control	0.96	11.0
6	Potassium phosphate	0.112%	Conventional control	0.06	86.9
7			Conventional control	1.72	90.0
8			No control	0.73	173.0

Note) The concentration of the active ingredient A is the concentration converted into  $P_2O_5$ .

5 Note) The grains colored with scarlet or orange inherent for Fusarium head blight and shrink grains were sorted as the diseased grain, and the grains other than them were sorted as the healthy grains.

10 The concentration of DON contamination of the healthy grains varied almost within lower range. On the other hand, in the diseased grains with Fusarium head blight, DON was detected at an extremely high concentration as expected. However, in the crude wheat determined to be the diseased  
 15 grains, difference was observed in the concentration of DON contamination. That is, in the section given the treatment with potassium phosphite, the DON contamination concentration was decreased depending on the given concentration of potassium phosphite. This way, it is  
 20 obvious that giving potassium phosphite reduces the DON contamination concentration in the wheat regardless the presence or absence or the degree of the disease with

Fusarium head blight of cereals, and further that the reduced degree of the DON contamination depends on the given concentration of potassium phosphite.

5 Example 3: Mixed use (1)

Wheat (cultivar: Haruyutaka) was seeded on April 23, 2002, and cultivated according to a conventional cultivating standard (Hokkaido, Agriculture Department, 1995) to establish test sections with 10 m<sup>2</sup> per section (3  
10 repeats). A suspension containing potassium phosphite and various fungicidal agents was prepared in a liquid mixture made up of a spreading agent (Gramine S: supplied from Hokkai Sankyo Co., Ltd.) and water. Using potassium phosphite as the compound A, 0.070% P<sub>2</sub>O<sub>5</sub>, and the 0.006 to  
15 0.025% fungicidal agent for agri-horticulture for the wheat as the compound B, water solution containing them was prepared, and 100 L thereof per 10 a was sprayed on leaves in the following growth stages. That is, the first spray (June 24, heading date), the second (June 30, flowering  
20 stage), the third (July 9) were performed. The harvest was performed on August 12 (50 days after the heading date) by harvesting the wheat in 3.4 m<sup>2</sup> in each section. After the harvest, the wheat was applied on a rice grader with 2.2 mm opening of screen to make selected grains, which were then  
25 pulverized by a rapid pulverizer to make whole grain powder. The whole grain powder was used as a sample for analysis. The DON contamination concentration was analyzed using HPLC-UV method in officially fixed methods shown by the Minister of Health, Labor and Welfare. Quantitative  
30 analysis was performed by repeating three times, and the quantitative value was an average value thereof.

Preparation of analysis sample solutions and the analysis procedure are briefly described.

- To 50 g of the whole grain powder, 85% acetonitrile  
35 was added, vigorously agitated for 30 min, and subsequently sonicated for 10 min.

- After eliminating impurities with filter paper, a filtrate was purified using a pretreatment column MultiSep #227 to make a solution for analysis.

5     - The test solution for analysis was injected into high performance liquid chromatography, and DON was detected by ultraviolet rays.

- The DON concentration of each test solution was read out from a standard curve made using DON standard solutions.

10     - The incidence rates of Fusarium head blight for panicles and spikelets were surveyed for 100 panicles in each section.

The results of the present test are shown in Table 3.

Table 3. Mixed use effects of potassium phosphite and various fungicidal agents

	Presence or absence of active ingredient A, potassium phosphite	Active ingredient B, Concentration of active ingredient	DON concentration (ppm)	Incidence rate for panicles (%)	Number of diseased spikelets/ panicle
1	×	Tebuconazol 0.020%	0.39	0.3	0.003
2	○		0.38	1.0	0.010
3	×	Metconazol 0.006%	0.34	0.7	0.007
4	○		0.10	1.0	0.010
5	×	Propiconazol 0.017%	1.01	1.0	0.010
6	○		0.72	2.7	0.027
7	×	Azoxystrobin 0.010%	4.65	2.3	0.027
8	○		0.96	2.0	0.023
9	×	Kresoxim- methyl 0.021%	1.05	0.3	0.003
10	○		0.51	0.7	0.007
11	×	Iminoctadine acetate 0.025%	1.04	1.7	0.017
12	○		0.20	3.0	0.030
13	○		0.77	4.3	0.050
14	Non-treatment		3.69	6.7	0.138

Note) 0.07% P<sub>2</sub>O<sub>5</sub> as potassium phosphite; circle: treated, crisscross: untreated

5 Note) The incidence rates for panicles and spikelet rates were surveyed for 100 panicles in each test section.

#### Example 4: Mixed use (2)

10 Wheat (cultivar: Haruyutaka) was seeded on May 4, 2003, and cultivated according to a conventional cultivating standard (Hokkaido, Agriculture Department, 1995) to establish test sections with 10 m<sup>2</sup> per section (3 repeats). A suspension containing potassium phosphite and

various fungicidal agents was prepared in a liquid mixture made up of a spreading agent (Gramine S: supplied from Hokkai Sankyo Co., Ltd.) and water. Using potassium phosphite as the compound A, 0.070%  $P_2O_5$ , and the 0.030 to 5 0.125% fungicidal agent for agri-horticulture for the wheat as the compound B, an water solution containing them was prepared, and 100 L thereof per 10 a was sprayed on leaves in the following growth stages. That is, the first spray (July 1, flowering stage), the second (July 7), the third 10 (July 14) were performed. Harvest was performed on August 25 by harvesting all wheat in each test section. After the harvest, using a sample divider, the wheat was equally divided, and was applied on a rice grader with 2.2 mm opening of screen to make selected grains, which were then 15 pulverized by a rapid pulverizer to make whole grain powder. The whole grain powder was used as a sample for analysis. The concentration of DON contamination was analyzed by the ELISA method in the same way as shown in Example 1.



Table 4. Mixed use effects of potassium phosphite and various fungicidal agents

	Presence or absence of active ingredient A, potassium phosphite	Active ingredient B, Concentration of active ingredient	DON concentration (ppm)	Incidence rate for panicles (%)	Number of diseased spikelets/ panicle
1	×	Tebuconazol 0.020%	0.16	7.0	7.3
2	○		0.08	5.0	5.0
3	×	Propiconazol 0.017%	0.57	4.7	5.0
4	○		0.11	4.7	5.0
5	×	Azoxystrobin 0.010%	0.83	4.3	4.7
6	○		0.50	3.7	4.0
7	×	Trifloxistrobin 0.025%	0.35	4.3	5.0
8	○		0.12	3.0	3.0
9	×	Iminoctadine albesilate 0.030%	0.22	7.3	8.0
10	○		0.00	2.7	2.7
11	×	sulfur 0.125%	1.85	8.7	10.3
12	○		0.43	8.7	8.7
13	○		0.57	10.3	11.0
14	Non-treatment		2.15	20.4	23.8

Note) Potassium phosphite is 0.070% in  $P_2O_5$ . ○:with treatment, ×:without treatment

5 Note) The incidence rates for panicles and spikelets were surveyed for 100 panicles in each test section .

10 In the same way as shown in Example 3, in the mixed use with any of the fungicidal agents for agri-horticulture, both the incidence rate for panicle and the incidence rate for spikelet exhibited high values compared to a single treatment, but the DON contamination amount was reduced.

Worthy of special mention is the result in section given the single agent treatment with the compound A, where the disease rate of Fusarium head blight was high, nonetheless, the DON contamination amount was at a low level.

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Example 5: Effects on bacterial growth and toxin production  
(1)

A water solution containing 5.600% potassium phosphite was prepared, soaked into gas-sterilized wheat  
10 seeds of "Hokushin" , and subsequently Fusarium head blight pathogenic fungus of the cereals, *Fusarium graminearum* having DON production capacity was inoculated, which were then cultured at 27°C. On days 7, 14, 21 and 28 in the culture, the amount of Fusarium head blight pathogenic  
15 fungus of the cereals and the amount of DON production present in wheat grains were analyzed.

The preparation of ergosterol analysis sample solutions and the analysis procedure are briefly described.

- To 5 g of the culture, 80 ml of ethanol was added,  
20 and pulverized using a rapid pulverizer.

- The ethanol solution containing the pulverized culture was vigorously agitated for 30 min to extract ergosterol.

- After eliminating impurities with filter paper, a  
25 filtrate was concentrated under reduced pressure, dried, solidified and dissolved again in 10 ml of ethanol to make an analysis sample solution.

- The analysis sample solution was injected into high performance liquid chromatography, and ergosterol was  
30 detected by ultraviolet rays.

The preparation of DON analysis sample solutions and the analysis procedure are briefly described.

- To 4 g of the culture, 80 ml of distilled water was added, and pulverized using a rapid pulverizer.

35 - The water solution containing the pulverized culture was vigorously agitated for 30 min to extract DON.

- A part of the extract solution was centrifuged, and the supernatant was used for the ELISA analysis.

- According to the instruction manual in the ELISA kit, various reagents were added, and subsequently an absorbance of each test solution was measured.

- The DON concentration of each test solution was read out from standard curve made using DON standard solution.

10 Table 5. Change over time of bacterial growth and DON production in wheat treated with potassium phosphite

Treatment	Analysis item	Days of culturing			
		7 days	14 days	21 days	28 days
Potassium phosphite 5.6%	DON (ppm)	ND.	ND.	ND.	ND.
	Ergosterol peak area value	17952	115921	372377	350972
Non-treatment	DON (ppm)	6.3	4.43	4.29	37.8
	Ergosterol peak area value	150453	271882	367236	559014

Note) ND means that the analytical value is less than the detection limit (0.222 ppm) of the ELISA analysis kit .

15 The amount of ergosterol which was an indicator of the amount of bacteria was continuously increased during the culture in the non-treatment whereas reached a plateau on the 21st day in the treatment with potassium phosphite. With respect to the amount of DON production, in the non-treatment, the DON production was initiated at an early phase of the culture and remarkably increased on the 28th day. On the contrary, in the treatment with potassium phosphite, DON was not detected during the entire period of the culture. It is obvious that application of potassium phosphite has a high inhibitory effect on the DON production regardless of the presence or absence or the

proliferation degree of the bacteria for the Fusarium head blight of cereals.

Example 6: Effects on bacterial growth and toxin production

5 (2)

A water solution containing 0.056% to 2.800% potassium phosphite was prepared, soaked into gas-sterilized wheat seeds of "Hokushin", and subsequently Fusarium head blight pathogenic fungus of the cereals, *Fusarium graminearum* having DON production capacity was inoculated to the wheat seeds, which were then cultured at 27°C. On day 28 in the culture, the amount of Fusarium head blight pathogenic fungus of the cereals and the amount of DON production present in wheat grains were analyzed by the methods in the same way as shown in Example 5.

Table 6. Effects of potassium phosphite concentration on bacterial amount of Fusarium head blight pathogenic fungus of the cereals and the amount of DON production

Treatment concentration	DON (ppm)	Ergosterol peak area
0.056%	1.60	1834861
0.112%	0.41	1769582
0.028%	ND.	1810454
0.560%	ND.	1921693
2.800%	ND.	85891
Non-treatment	46.1	3601748

20 Note) ND means that the analytical value is less than the detection limit (0.222 ppm) of the ELISA analysis kit.

Considering the amount of ergosterol as the indicator of the amount of bacteria, in the treatment with the water solution of 0.056 to 0.560% potassium phosphite, the inhibitory effect on the bacterial amount at a moderate

degree and similar degree as compared to that in the non-treatment (water only) was observed, and the remarkable inhibitory effect on the bacterial amount was observed in the treatment with the water solution of 2.800% potassium phosphite. Concerning DON amount, in the range of concentrations from 0.056 to 2.800% of potassium phosphite in the water solutions, it was significantly lower than that in the non-treatment. It is obvious that the application of potassium phosphite has a high inhibitory effect on the DON production regardless of the presence or absence or the proliferation degree of the bacteria for the Fusarium head blight of cereals.

#### Industrial Applicability

According to the invention, it has been demonstrated that an excellent inhibitory effect on mycotoxin contamination, particularly DON contamination can be had regardless of the presence or absence or the degree of the disease with Fusarium head blight of cereals although the prevention effect on Fusarium head blight pathological fungi of cereals is low by spraying one or more compounds A selected from the group consisting of ammonium salts, primary to quaternary ammonium salts, alkali metal salts, alkaline earth metal salts and polyvalent metal salts of phosphorous acid and phosphite ester onto the growing wheat. Also, it has been demonstrated that the contamination amount of mycotoxin is further reduced by the mixed use of the other fungicidal agent for agri-horticulture compared to the use of the single fungicidal agent alone.